Effect of 2-chloroethylphosphonic acid on capsule formation and alkaloid content in Papaver somniferum

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Application of different concentrations of ethephon (2-chloroethylphosphonic acid) to *Papaver somniferum* L. at the times of stem elongation, bud, and capsule formation produced different effects. Ethephon (10^{-2} *M*) retarded growth of the plant and inhibited capsule formation during stem elongation, significantly reduced capsule size during the flowering period, but did not alter capsule development during capsule formation. When applied during the period of stem elongation, ethephon (10^{-3} *M* and 10^{-4} *M*) reduced capsule size; alkaloid accumulation was reduced by ethephon at a concentration of 10^{-3} *M*, but slightly increased by 10^{-4} *M*. Ethephon (10^{-3} *M* and 10^{-4} *M*) did not alter capsule development or alkaloid content significantly when applied during bud formation, but stimulated capsule size and alkaloid content when applied during capsule formation. Pretreating the plants with Ag^+ (silver nitrate) did not reverse the ethephon effect. The results suggest that capsule maturation and alkaloid accumulation in *P. somniferum* are modified by ethylene, which is produced as a result of exogenous ethephon treatment.

Additional key-words: Ethephon, capsule maturation, alkaloid accumulation.

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Introduction

Ethylene plays a major role in regulation of fruit ripening, senescence of flowers and leaves, abscission, growth inhibition, tissue proliferation, root growth inhibition, geotropism and epinasty (Pratt and Goeschl 1969, Kende and Hanson 1976, Aharoni and Lieberman 1979). A water soluble slow ethylene-releasing growth regulator "Ethephon" (2-chloroethylphosphonic acid) decomposes spontaneously in aqueous solution and in tissues to yield ethylene (Cooke and Randell 1968, Morgan 1969, Bache 1970).

Generally, the effect of ethylene is opposed by effects of other growth hormones-auxins, cytokinins, and gibberellins. These hormones, alone or in combination, can, in several instances, induce ethylene production (Lieberman and Kunishi 1971). Ag^+ opposes the effect of ethylene, presumably by blocking ethylene action at its receptor site (Aharoni *et al.* 1979).

Our research objective is to seek means to control the production of morphinan alkaloids (MA's) in *P. somniferum*. The biosynthesis of morphinan alkaloids is largely through enzymatic reactions. Ethylene has been implicated in the control of de novo enzyme synthesis (Moore 1979). The formation of secondary metabolites in the potato tuber has been shown to be regulated by exogenous ethylene (Alves *et al.* 1979). The aim of this study was to investigate the possible role of ethylene in the production of morphinan alkaloids. We also probed the interaction between Ag^+ and ethephon in terms of alkaloid synthesis and plant growth.

Abbreviations: P. somniferum, *Papaver somniferum* L.; IAA, indole-3-acetic acid; NAA, 1-naphthylacetic acid; GA_3, gibberellic acid; HPLC, high performance liquid chromatography; MA, morphinan alkaloid.

Materials and methods

Seeds of *Papaver somniferum* L. were provided by Dr. Quentin Jones, Agricultural Research, Science and Education Administration, US Department of Agriculture, Beltsville, Maryland, USA. Ethephon and detergent, Tween 80 (polyoxyethylene sorbitan monooleate), were from Sigma Chemical Co.; IAA, NAA, and GA_3 were from Gibco Biological Co. Solvents for HPLC and extraction of alkaloids were from commercial sources and were Mallinckrodt Nanograde, or equivalent. Ag^+ in the form of silver nitrate was from J. T. Baker Chemical Co.

Growth of plants. Seeds of P. somniferum were planted in sterilized sandy loam. The plants were grown in an Environmental Growth Chamber (Model M-2) under previously described conditions (Tookey *et al.* 1976). Seedlings were thinned to two plants per pot at 3 weeks of age, and fertilizer was applied every other week for 10 weeks at one-half the concentration applied once per month by Tookey *et al.* Flowering was initiated by increasing photoperiod from 8 to 14 h after 10 weeks.

Treatment of plants with ethephon, IAA, NAA, GA_3, and Ag^+. Plants were treated by spraying with the following reagents: (a) ethephon (10^{-2} *M*, 10^{-3} *M*, 10^{-4} *M*); (b) IAA (10^{-5} *M*); (c) NAA (10^{-5} *M*); (d) GA_3 (10^{-5} *M*); (e) Ag^+ (10^{-5} *M*, 10^{-4} *M*, 10^{-3} *M*) in distilled water containing 0.5% of non-ionic detergent (Tween 80). The control plants were sprayed with distilled water containing only detergent. All plants were sprayed to the point of run off. When the treatment involved more than one reagent, the plants were allowed to dry before they were sprayed with the other reagent(s). Plants were treated with the above reagents with either a single spray application or three separate applications. For three-application experiments, the plants were sprayed at 3-day intervals. The single applications were made at the same time as the second spraying of the three-application experiments. Treatments with the above reagents were made at three periods during plant growth as follows: (a) Stem elongation: treatment began 10 days after the photoperiod was increased from 8 to 14 h. (b) Bud formation: treatment began the first day of flowering. (c) Capsule development: treatment began 2–5 days after the flower petals dropped. All the treatments were replicated once.

Extraction and analysis of morphinan alkaloids. Capsules from different experiments were freeze-dried, and the dried capsules (with seeds) were pulverized in a ball mill (Prolabo, Microbrogeur Quantitatif Dangoumau). One gram of the dried, powdered capsule material was added to 15 ml of 5% acetic acid, sonicated and adjusted to pH 9.0 with concentrated NH_4OH, and then extracted three times with 20 ml portions of chloroform-isopropanol (3:1, v/v). The combined chloroform extracts were dried over anhydrous sodium sulfate, then evaporated to dryness under a nitrogen stream. The dried residue was dissolved in 1 ml of absolute ethanol. An aliquot of the ethanol solution was analyzed for thebaine, codeine, and morphine by HPLC with minor mocification of the procedure of Vincent and Engelke (1979). An HPLC (Waters Associates) was equipped with a (Perkin Elmer) stainless steel column (30 cm × 3.9 mm i.d.) packed with 5 μm porous silica gel, a multiple wavelength UV-visible detector (Waters Associated, Model 450 variable wavelength detector) and a syringe-loading injector with 100 μl capacity. The solvent was *n*-hexane:chloroform:ethanol:diethylamine (300:30:40:0.5). Conditions for chromatography were as follows: column temperature, 25°C; flow rate, 2 ml/min at (7–10) × 10^5 N/cm^2; detector wavelength, 285 nm.

Treatment of plants with ethylene. Two clear glass cylinders equipped with entrance and exit ports were placed in the environmental growth chamber. The control plants in one of the glass cylinders were constantly flushed with air, while the plants to be treated were flushed with ethylene (10 μl/l in air). The treatment was continued up to 1 week.

Results

When the photoperiod in the environmental growth chamber was increased from 8 h to 14 h, plants of P. somniferum started to elongate. Plants grew rapidly during this period, and flowering was initiated. Figure 1 shows the effect on capsule size when different concentrations of ethephon were sprayed on the plants three times during this period. Plants treated with 1.4 × 10^{-2} *M* ethephon exhibited no capsule formation and

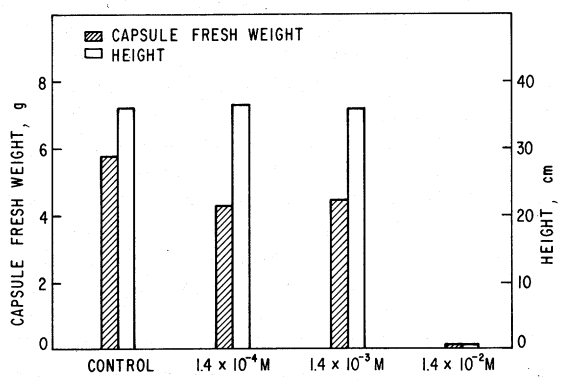

Fig. 1. Effects of increasing concentrations of ethephon on capsule development during the stem elongation period of growth of *Papaver somniferum.* Plants were treated with ethephon three times as described under "Materials and methods". Capsules were harvested 10 days after petal drop. Each treatment represents eight capsules. Standard deviation is no more than 5%.

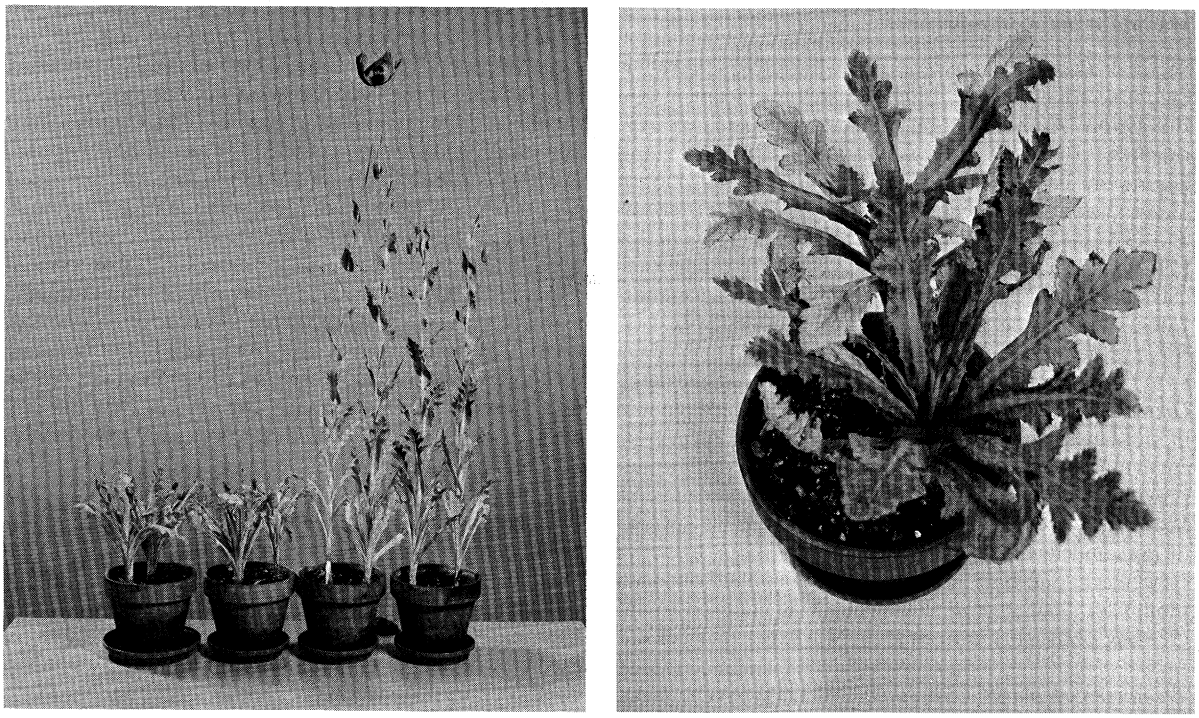

Fig. 2. Comparison of normal and ethephon-treated plants of *Papaver somniferum*. (A) Two plants sprayed once with 1.4×10^{-2} *M* ethephon (at left) compared to two non-treated plants (at right). (B) Atrophy of flower bud on an ethephon-treated plant.

their growth rate was markedly reduced. Lower concentrations of ethephon (1.4×10^{-3} *M* and 1.4×10^{-4} *M*) only reduced capsule size by 20% as compared to the control capsules. The morphology of plants treated with 10^{-3} *M* and 10^{-4} *M* of ethephon was essentially identical to that of the control plants. Figure 2A shows a comparison of normal plants (with capsule formation) and plants sprayed once with 10^{-2} *M* ethephon (without capsule formation). A close-up view of the top of the treated plants (Fig. 2B) shows that the flower buds atrophied without capsule development. As indicated above, the strong growth-inhibiting effect of 1.4×10^{-2} *M* ethephon occurred when applied during the stem elongation period of P. somniferum. Similar treatments were carried out during bud formation and capsule development periods (Fig. 3). Treatment of plants with 1.4×10^{-2} *M* ethephon during the bud formation caused the capsules to be reduced to 30% the size of capsules formed on non-treated plants, and leaf abscission was 60% more frequent. When the ethephon treatment was administered at the capsule development stage, no apparent change in capsule size or other morphological alteration occurred.

The effects of ethephon (10^{-3} *M* and 10^{-4} *M*) on the capsule size and MA accumulation of P. somniferum at the different growth periods are summarized in Table 1. Relative to control plants, accumulations of thebaine, codeine, and morphine are reported in the aggregate under the general heading of MA's in Table 1; increases or decreases of the individual MA's in the capsules of plants treated with ethephon were proportional to changes in the total accumulation of MA's. The typical individual accumulations of thebaine, codeine and morphine in the capsules of control plants are provided in Table 2. When applied during the stem elongation

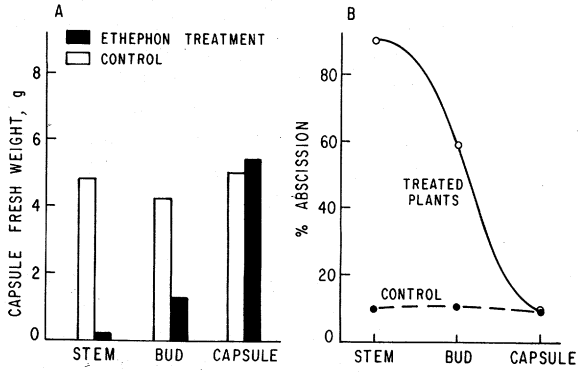

Fig. 3. Effect of ethephon (10^{-2} *M*) on (A) capsule development and (B) leaf abscission of *Papaver somniferum* during different growth periods. Ethephon (1.4×10^{-2} *M*) was sprayed on the plants during the periods of stem elongation, bud formation, and capsule development. Each data point represents an average of six plants. Legend as for Figure 1.

Tab. 1. Effect on plant growth and morphinan alkaloid (MA) accumulation by treatment with 10^{-3} *M* and 10^{-4} *M* of ethephon. Plants were sprayed three times during the periods of stem elongation, bud formation, or capsule development. Data expressed as a percentage of control.

Period of treatment	Ethephon (10^{-4} *M*)		Ethephon (10^{-3} *M*)	
	Capsule (dry wt.)	MA's	Capsule (dry wt.)	MA's
Stem elongation	87	110	78	79
Bud formation	93	113	104	106
Capsule development	140	108	141	114

period, the degree of retardation of plant growth was proportional to the ethephon concentration used. Treatment with ethephon at 10^{-3} *M* decreased the capsule size approximately 20%, and the accumulation of MA's decreased in proportion. Ethephon treatment at 10^{-4} *M* decreased the capsule size, while MA accumulation was slightly increased. During the bud formation period, treatment with ethephon at 10^{-3} *M* slightly increased both the capsule size and MA content. Ethephon at 10^{-4} *M* slightly decreased the capsule size, while MA accumulation was increased. During the capsule development period, treatment with both 10^{-3} *M* and 10^{-4} *M* concentrations of ethephon increased the capsule size significantly, but did not increase the MA content in proportion to the capsule size.

Ag^+ is known to oppose the effect of ethylene, presumably by blocking ethylene action at its receptor sites (Beyer 1978, Burg and Burg 1965). Increasing concentrations of Ag^+ from 10^{-5} to 10^{-3} *M*, with or without ethephon treatment (Fig. 4), were tested for their effects on the growth of P. somniferum. With increasing Ag^+ concentration, the capsule size gradually diminished. Treatment with 10^{-4} *M* Ag^+ reduced capsule size up to 50%, and 10^{-3} *M* Ag^+ completely retarded capsule formation. Ag^+ at 10^{-6} *M* had no apparent effect on either the capsule size or plant growth. Leaf abscission occurred only when plants were treated with 10^{-3} *M* Ag^+. Pretreatment of plants with all concentration of Ag^+ prior to ethephon (1.4×10^{-2} *M*) treatment did not prevent the effect of ethephon.

Some other plant hormones were also tested for their effects on the growth and alkaloid accumulation of P. somniferum. Aharoni *et al.* (1979) showed that IAA and kinetin can stimulate endogenous ethylene production at concentrations between 10^{-5} *M* and 10^{-6} *M* in aging tobacco leaf discs. IAA, NAA, and GA_3 at 10^{-5} *M* were applied individually to P. somniferum during the stem elongation period in three spraying treatments. Table 2 shows that IAA had a stronger growth inhibitory effect than GA_3, NAA, and Ag^+ (from Fig. 4) at similar concentrations. IAA markedly reduced thebaine, codeine, and morphine accumulation, GA_3 did not significantly alternate the content of thebaine, codeine and morphine, while NAA increased both thebaine and morphine, but not codeine.

When plants of P. somniferum were treated with

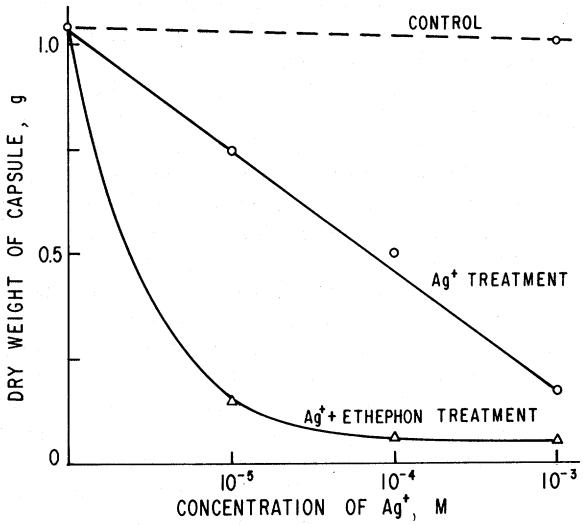

Fig. 4. Effect of increasing concentrations of Ag^+ on the capsule growth of *Papaver somniferum.* Different concentrations of Ag^+ were applied to P. somniferum with or without treatment with ethephon (1.4×10^{-2} *M*). Capsules were harvested 10 days after petal drop. Each data point represents four capsules. Legend as for Figure 1.

Tab. 2. Effect of exogenous hormones on capsule development and morphinan alkaloid accumulation. Plants were sprayed three times with hormones during the period of stem elongation and were harvested 10 days after petal drop. Alkaloids were extracted from the capsules and analyzed by high performance liquid chromatography. Each treatment, once replicated, represents four capsules.

Treatment	Capsule g fresh wt	Plant height cm	Morphinan alkaloids, mg/capsule		
			Thebaine	Codeine	Morphine
Control	5.82±0.30	36±2.0	1.60±0.08	21±1.0	322±13
IAA (10^{-5} *M*)	3.10±0.16	18±1.0	1.20±0.06	14±0.75	228±13
NAA (10^{-5} *M*)	5.16±0.30	30±2.0	1.90±0.10	18±1.1	367±18
GA_3 (10^{-5} *M*)	4.58±0.25	31±2.0	1.70±0.09	20±1.2	314±16

ethylene (10 µl/l in air) during the stem elongation period, the leaves of treated plants were abscissed 20 to 30% more than the control plants after 72 h. Plants that were given an additional 48 h of ethylene treatment, and then allowed further growth for one week without ethylene did not produce capsules, despite their otherwise normal appearance. These results and the results of some other studies (Morgan 1969) indicate that the action of ethephon is through ethylene function.

Discussion

This is the first published report that ethephon treatment inhibits capsule formation and alkaloid accumulation in P. somniferum. These effects depend primarily on the concentration of ethephon and the growth stage of the plant. It is obvious that ethephon treatment can modify capsule maturation, which in turn controls the morphinan alkaloid production in P. somniferum. In fact, this ability may be useful for controlling morphine production in P. somniferum.

Ethephon treatment of P. somniferum also inhibits plant growth and bud formation as reported for other plants (Burg and Burg 1966). These authors showed the classic inhibitory effect of superoptimal concentration of auxin (in the case of dicotyledonous stem segments) by the auxin-induced production of ethylene, and more recently, a similar function of ethylene in the auxin induced inhibition of bud growth. Ethephon has been noted for its ability to induce premature abscission of leaves and other organs. If this induction is caused exclusively by ethylene resulting from the application of ethephon, the abscission response should be blocked by Ag^+ or exogenous auxin (Aharoni *et al.* 1979). Hall (1952) demonstrated that the abscission activity of ethylene is reduced or blocked completely if plants are first treated with auxin. In our study, pretreatment of P. somniferum with all concentrations of Ag^+ tested did not block the action of ethylene as occurred in other studies with tobacco leaf discs (Aharoni *et al.* 1979) wherein treatment with Ag^+ at $6 \times 10^{-5} M$ opposed the effect of ethylene. The failure to observe inhibition in the present study may be related to a penetration problem. In our study, low concentrations of Ag^+ did not oppose the effect of ethephon, but higher concentrations of Ag^+ reduced capsule size, possibly by simple toxic effects. The combination of Ag^+ and ethephon treatment would account for the acceleration of the growth inhibition effect. We also found that $10^{-5} M$ of NAA, IAA, or GA_3 applied prior to treatment with ethephon did not prevent abscission by ethephon. However, the results of the ethylene experiment suggest that the effects of ethephon on the leaf abscission and capsule maturation of P. somniferum are due to the action of ethylene.

Acknowledgements – Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

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